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The response of $\gamma\delta$ T cells to *Plasmodium falciparum* is dependent on activated CD4$^+$ T cells and the recognition of MHC class I molecules

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SUMMARY

Peripheral blood $\gamma\delta$ T cells from non-exposed individuals respond to antigens of the malaria parasite, *Plasmodium falciparum, in vitro*. This response, largely caused by T cells bearing the Vγ9$^+$ chain of the T-cell receptor, is stimulated by components of the parasite expressed on the schizont stage and released at schizont rupture. The response of Vγ9$^+$ T cells to parasite components is inhibited by antibodies to major histocompatibility complex (MHC) class I and class II. However, the inhibition by anti-MHC class II antibodies can be overcome by the addition of interleukin-2 (IL-2) to the cultures, suggesting that $\gamma\delta$ T cells themselves do not recognize MHC class II molecules but require an MHC class II-dependent response taking place in the culture. In contrast, the inhibition by anti-class I antibodies cannot be reversed by addition of IL-2. Since an accompanying CD4$^+$ T-cell response occurred in peripheral blood mononuclear cells cultured with *P. falciparum* antigens, it was considered that these cells provide the cytokines necessary for the subsequent activation and expansion of Vγ9$^+$ T cells recognizing components of the parasite and MHC class I molecules. This was confirmed by reconstituting the response of enriched $\gamma\delta$ T cells to *P. falciparum* schizont extract by addition of purified CD4$^+$ T cells.

INTRODUCTION

T lymphocytes bearing heterodimeric T-cell receptors composed of γ and δ chains ($\gamma\delta$ T cells) have been proposed to represent a primitive immune surveillance system. Evidence for this is largely circumstantial, but is supported by the demonstration of $\gamma\delta$ T-cell responses to a wide variety of different infections.[1–7] In many animals $\gamma\delta$ T cells demonstrate both preferential epithelial localization and site-dependent T-cell receptor Vγ and Vδ chain usage.[8] In humans, $\gamma\delta$ T cells are a minority of peripheral blood T lymphocytes[9] and within this small subpopulation cells bearing Vγ9 and Vδ2 chains of the T-cell receptor (TCR) predominate. During life they increase within the $\gamma\delta$ T-cell pool from 25% in cord blood to 70–90% in adult peripheral blood.[8] Concomitant with this increase is the expression of CD45RO,[10] suggesting that such expansion is the result of repeated encounters with antigen.

Among the infectious diseases where $\gamma\delta$ T-cell expansion has been reported, *Plasmodium falciparum* malaria is of particular interest.[4,5,11] In addition to the increased level observed in the peripheral blood of non-immune individuals during and after a malaria episode,[4,5,11] there is a remarkable *in vitro* proliferation of Vγ9$^+$/Vδ2$^+$ $\gamma\delta$ T cells from naive individuals in response to *P. falciparum* antigens.[12–14] The response of $\gamma\delta$ T cells may be beneficial to the host, as they have been shown to exert direct anti-parasitic activity.[15] Tumour necrosis factor(s) (TNF) and interferon-γ (IFN-γ) produced by $\gamma\delta$ T cells in response to parasite-infected red cells[16,17] may promote macrophage activation that also could result in parasite killing. However, pro-inflammatory cytokines such as TNF and IFN-γ have been implicated in the pathology of malaria,[18,19] and the large response to malarial antigens observed *in vitro* may represent an excessive and thus pathological response *in vivo*.

The antigen specificity of $\gamma\delta$ T cells in malaria has not been defined. Preliminary studies have shown that the response is reduced when allogeneic instead of autologous antigen-presenting cells (APC) are used[20] and when antibodies to either major histocompatibility complex (MHC) class I or class II are introduced.[13] Parasite material from the schizont stage of cultured *P. falciparum* has been found to be the most potent source of antigen with which to stimulate human $\gamma\delta$ T cells.[17] The strict rules governing recognition of peptides and MHC class I or class II molecules by CD8$^+$ and CD4$^+$ T cells seem not always to apply in the case of $\gamma\delta$ T cells. A few human and mouse $\gamma\delta$ T-cell clones do exhibit specificity for antigens presented on MHC class I or II, and some epitopes of heat-shock proteins have been defined.[8] However, there is evidence to suggest that MHC class I-like proteins such as CD48 and the

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Abbreviations: Ab, antibody; Ag, antigen; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor.

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CD1 family of molecules may also be recognized.[21] The ability of some of these molecules to present non-peptide material[22,23] and the recognition of monoalkylphosphate and phenyl pyrophosphate derivatives of mycobacteria by some $\gamma\delta$ T cells[24] raise the possibility that the $\gamma\delta$ T-cell response in malaria may be similarly stimulated.

In this study we have examined some of the requirements for the response of $\gamma\delta$ T cells to *P. falciparum in vitro*. Inhibition studies with a panel of antibodies to MHC class I and class II suggest that the $\gamma\delta$ T-cell response to *P. falciparum* antigens is dependent upon a concomitant MHC class II-restricted response of $CD4^+$ T cells taking place in the culture. This dependency can be replaced by the addition of exogenous IL-2, and the $V\gamma9^+$ T-cell response itself recognizes antigens presented on MHC class I molecules.

MATERIALS AND METHODS

Donors and mononuclear cell isolation

Blood donors were healthy European volunteers with no history of exposure to malaria. Peripheral blood mononuclear cells (PBMC) were prepared by centrifugation over Ficoll–Hypaque (Pharmacia, Freiburg, Germany).

Parasite culture

The K1 strain of *P. falciparum* (Hughes Matille, Hoffmann-La Roche, Basel, Switzerland) was cultured by standard methods. Supernatants were obtained from cultures at the time of schizont rupture and passed through a 0·2μ filter before use as a source of malarial antigen.[17]

Culture of PBMC with P. falciparum *antigen*

PBMC were cultured in 96-well microtitre plates (10^5 cells in a total volume of 200 μl) in RPMI-1640 (Gibco-BRL, Paisley, UK) containing 2 mM L-glutamine (Gibco), 20 mM HEPES (Gibco), penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively; Gibco) and 10% heat-inactivated human AB serum, for 5–6 days in the presence of *P. falciparum* culture supernatants (used at a final concentration of 10%).

In order to determine whether antibodies to MHC class I or class II molecules could inhibit the $\gamma\delta$ T-cell response, specific monoclonal antibodies and the appropriate isotype control antibodies were incubated with the PBMC in the microtitre wells for 2 hr at 37° prior to the addition of *P. falciparum* culture supernatants. The following antibodies were used: W6/32 (anti-HLA-A, -B, -C; Harlan Seralab, Crawley Down, Sussex, UK), BI-3D3 (anti-HLA-A, -B, -C; Harlan Seralab), 2A1 (anti-HLA class I monomorphic determinants; a kind gift of Professor P. Beverley, I.C.R.F., London, UK), L243 (anti-HLA-DR; ATCC, Rockville, MD), HL-40 (anti-HLA-DR, -DP; Exbio, Prague, Czech Republic), FMC14 (anti-HLA-DR; Harlan Seralab), and the relevant IgG1 and IgG2a isotype control antibodies (Sigma, St Louis, MO). Antibodies were used in culture at final concentrations of 0·5, 2·5 and 12·5 μg/ml. None of the antibodies used was non-specifically toxic in culture at any of the concentrations. They were tested prior to use on a panel of mouse and human tumour cell lines and found not to affect their growth and cell division. Where appropriate, cultures were supplemented with recombinant human IL-2 (a kind gift of Dr A. Lanzaveccia, Basel, Switzerland) at a final concentration of 10 U/ml. The magnitude of the response of PBMC was determined from the proportion of blasting cells as defined by flow cytometry (see below). The total number of blasting $V\gamma9^+$, $CD4^+$ and $CD8^+$ cells (see below) was then calculated from the total number of viable cells recovered from triplicate wells after 5–6 days of culture.

Flow cytometric analysis

After 5–6 days of culture, cells were harvested from the microtitre wells and the replicates pooled, counted, washed and resuspended in sorter buffer [phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) and 0·02% sodium azide]. Sequential three-colour staining was carried out using appropriate combinations of the following. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or biotin-labelled antibodies diluted in sorter buffer: anti-CD3 (Leu-4, Becton Dickinson, Oxford, UK; OKT3, ATCC), anti-CD4 (B-F5; ImmunoQuality Products, Bad Neuheim, Germany), anti-CD8 (UCHT4, a kind gift of Professor P. Beverley; MCD8, ImmunoQuality Products), anti-$V\gamma9$ (4D7; a kind gift of Dr K. Pfeffer), anti-$\gamma\delta$ T-cell receptor (TCRδ1; T Cell Diagnostics, Cambridge, MO), and appropriate isotype control antibodies (Becton Dickinson). Streptavidin Red 670 (Gibco) was used as the second-step reagent for the biotin-labelled antibodies. After staining the cells were fixed in 1% paraformaldehyde before analysis. Flow cytometry was carried out on a FACSscan (Becton Dickinson) and analysed using Cell Quest software (Becton Dickinson). The response of T cells was determined by assessing the proportion of blasting cells in the analysed populations using forward and 90° scatter. Absolute numbers of blasting cells for each response were then calculated from the known number of harvested viable cells. The proportions and total number of $\gamma\delta$ T cells, $CD4^+$ and $CD8^+$ cells within the blasting cell population were recorded.

Purification of adherent cells (APC), $\gamma\delta$ T cells and $CD4^+$ T cells

Adherent cells were used as a source of APC. They were obtained by incubation of PBMC (3×10^6 cells/ml in RPMI containing 10% human AB serum) in Petri dishes for 90 min. Non-adherent cells were poured off and the adherent layer subsequently washed to remove any residual non-adherent cells. The adherent cells were recovered by gently scraping the plates with sterile syringe plungers. For use as APC the adherent cells were irradiated (3000 rads).

Non-adherent PBMC were used as a source of $\gamma\delta$ and $CD4^+$ T cells (see below). $\gamma\delta$ T cells were enriched by negative selection in two stages. The first round of enrichment used antibodies and magnetic beads. For this, cells were incubated with monoclonal antibodies specific for $\alpha\beta$ TCR (BMA 031; Behring, Marburg, Germany), CD4 (BMA 040; Behring), CD8 (OKT8; ATCC), CD14 (OKM1; ATCC) and CD19 (BMA 0130; Behring) for 30 min on ice. The cells were then washed twice and incubated with magnetic beads coated with goat anti-mouse IgG (Paesel and Lorei, Frankfurt, Germany) at a ratio of 25 beads per cell. The labelled cells were removed by magnetic separation. The remaining cells were incubated with FITC-labelled anti-CD4 antibodies (ImmunoQuality Products), PE-labelled anti-CD16 and anti-CD8 (Becton Dickinson and ImmunoQuality Products, respectively) for 30 min on ice. Unstained cells were separated from PE- and FITC-labelled cells on a FACStar Plus (Becton Dickinson) after gating out

any remaining monocytes, macrophages and dead cells by 90° and forward light scatter. The sorted cells contained less than 0·5% CD8$^+$, CD4$^+$, CD14$^+$, CD16$^+$ and CD20$^+$ cells and greater than 70% γδ T cells, as determined using FITC-labelled anti-γδ TCR antibodies (TCRδ1; T Cell Diagnostics). The remaining cells could not be identified with the panel of anti-leukocyte antibodies described above.

CD4$^+$ T cells were purified by removal of CD8$^+$, CD14$^+$ and CD19$^+$ cells from non-adherent PBMC using goat anti-mouse IgG-conjugated magnetic beads as described above. The resultant cells were incubated with PE-labelled anti-CD16, anti-CD8 and FITC-labelled anti-γδ TCR antibodies. CD16$^-$, CD8$^-$ and γδ TCR$^-$ cells were isolated by cell sorting as described above. The purity of CD4$^+$ T cells obtained was greater than 99%.

Culture of γδ T cells, CD4$^+$ T cells and APC with P. falciparum *schizont antigens*

For culture, sorted γδ T cells (1×10^5/well) were incubated with the autologous adherent cells as APC (1×10^4/well) and different doses of autologous CD4$^+$ T cells (0–30 000/well) in the presence of 10% *P. falciparum* culture supernatant for 5–6 days. The magnitude of the response and the phenotype of responding cells was determined as described for the response of unfractionated PBMC.

RESULTS

The γδ T-cell response of a panel of European donors to late-stage antigens of *P. falciparum*

γδ T cells usually comprise between 0·5 and 5% of circulating peripheral blood lymphocytes in European populations.[9] This was the case for the 11 donors used in the experiments described here (data not shown). In agreement with previous observations[1,7] *in vitro* culture of PBMC from all donors with a soluble extract of *P. falciparum* schizonts resulted in expansion of γδ T cells bearing Vγ9 chains compared with unstimulated control cultures (Table 1). The responding population, defined as blasting cells using forward and 90° light scatter (side scatter) on the flow cytometer, was variable from donor to donor and represented 22–84% of all cells recovered from culture, 7·3–86% of which were Vγ9$^+$ T cells. CD4$^+$ T cells were also present in varying proportions in the blasted cell populations of the different donors (a representative flow cytomeric analysis of blasting and non-blasting cells is shown in Fig. 1). In general the larger the total blasting-cell response the greater the proportion of Vγ9$^+$ T cells contained within that population. Cultures of PMBC without *P. falciparum* antigen contained few blasting cells (5–10%) and showed no preferential increase in the proportion of Vγ9$^+$ cells, the proportions remaining similar to those observed in the starting populations (2–18% Vγ9$^+$ γδ T cells and 17·8–57% CD4$^+$ T cells) as described previously.[13,17]

Inhibition of Vγ9$^+$ T-cell response by anti-class II antibodies can be circumvented by addition of IL-2 to cultures

Addition of anti-MHC class II antibodies to cultures of PBMC and *P. falciparum* extracts had a profound dose-dependent effect on the overall blasting response to the parasite extract.

Table 1. Response of Vγ9$^+$ γδ T cells to *P. falciparum* schizont antigens *in vitro* of donors with no previous exposure to malaria

	Unstimulated PBMC		PBMC stimulated with *P. falciparum* schizont antigen		
	Within total cells*		Within blasting cells§		
Donor	% Vγ9$^+$†	% CD4$^+$†	% Blasts‡	% Vγ9$^+$†	% CD4$^+$†
58	4·4	56·6	68·8	75·5	4·3
62	2·2	49·8	79·7	86·6	6·6
20	2·2	55·3	39·4	41·0	34·2
22	7·7	25·5	19·8	25·4	25·9
40	2·5	38·2	20·8	10·0	36·0
42	4·7	28·5	21·2	28·2	32·5
48	1·9	41·4	21·4	7·3	37·1
49	1·7	ND	11·5	34·1	ND
52	18·8	ND	27·7	39·4	ND
71	4·8	ND	24·2	44·3	ND
79	2·2	17·8	28·8	57·8	17·6

* PBMC incubated without *P. falciparum* antigen.
† The percentage of Vγ9$^+$ and CD4$^+$ T cells was determined by flow cytometry.
‡ The percentage of blasting (responding) cells was determined by forward and large (90°) angle scatter using FACSscan.
§ The percentage of Vγ9$^+$ or CD4$^+$ T cells was determined within the gated blasted population.
ND, not determined.

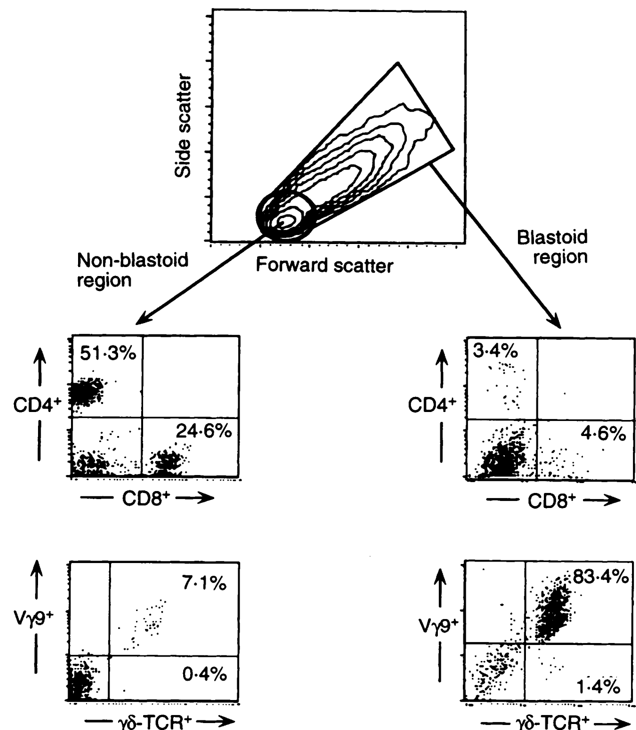

Figure 1. Flow cytometric representation of γδ T cells and CD4$^+$ T cells within the non-blasting and blasting cell populations after stimulation with *P. falciparum* antigens. PE-conjugated anti-CD4$^+$ antibodies and FITC-conjugated anti-Vγ9 antibodies were used to detect CD4$^+$ and γδ T cells, respectively, as described in the Materials and Methods.

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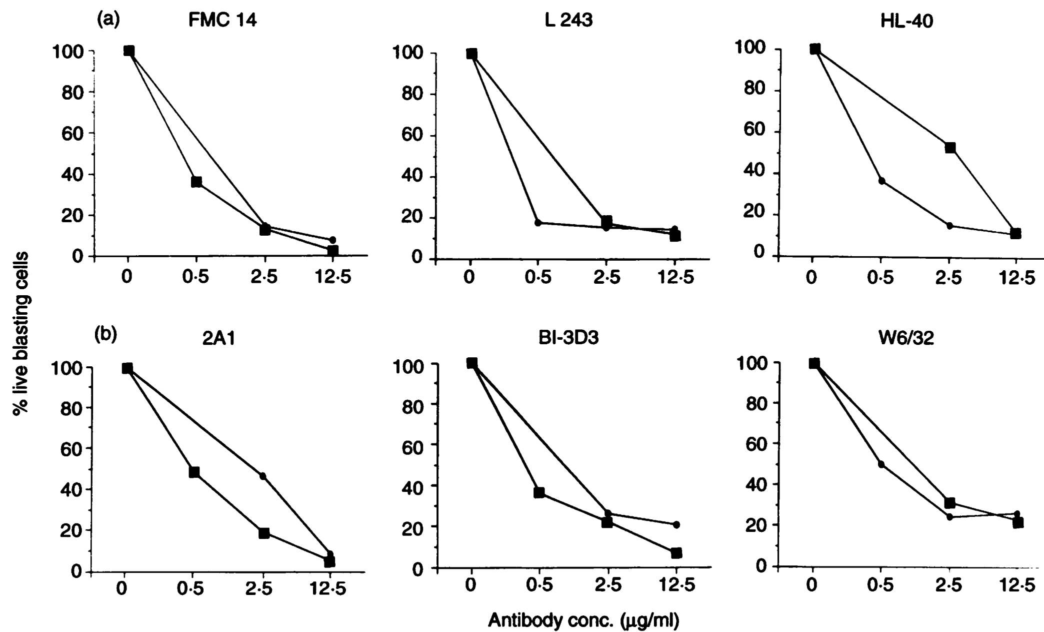

Figure 2. Dose-dependent inhibition of the blasting Vγ9$^+$ γδ T-cell response to *P. falciparum* antigens by antibodies specific for MHC class I and MHC class II. PBMC from two donors, 58 (■) and 62 (●), were cultured from *P. falciparum* culture supernatants in the presence of (a) anti-class II antibodies (FMC 14, L243, HL-40) and (b) anti-class I antibodies (2A1, BI-3D3, W6/32) at final concentrations of 0·5, 2·5 and 12·5 μg/ml. The results are expressed as a percentage of the total number of blasting cells recovered from cultures of PBMC, antigen and isotype-matched control antibodies at the same concentrations.

The total numbers and proportion of blasting cells recovered per well were substantially reduced in all donors tested (10 donors were tested). A representative experiment using different amounts of three different anti-MHC class II antibodies with a single donor is shown in Fig. 2a. All three antibodies reduced the percentage of total blasting cells (as determined by forward light scatter and 90° scatter using flow cytometry) by more than 60%. Addition of isotype-matched control antibodies of irrelevant specificity prepared in the same way did not affect the response to the parasite extract compared with control cultures without antibodies added (data not shown).

The reduction in the proportion of blasting cells was reflected in the total number of recovered viable Vγ9$^+$ T cells. This is shown in detail for two donors in Table 2. In cultures containing *P. falciparum* antigens, PBMC and control antibodies of irrelevant specificity, ≈2–6 × 10^5 viable blasted cells were recovered after culture (20–70-fold increase compared with cultures without antigen). Vγ9$^+$ T cells represented 71–88% of this blasting cell population. In the presence of 12·5 μg/ml of the three different anti-MHC class II antibodies, the total numbers of blasting Vγ9$^+$ T cells were reduced by more than 20-fold (1 × 10^3–2·4 × 10^4 cells).

As there was also a variable number of CD4$^+$ T cells responding in cultures stimulated with *P. falciparum* antigens (Tables 1 and 2), it is possible that cytokines produced from this MHC class II-restricted response permit Vγ9$^+$ T-cell proliferation. If this response is inhibited by the anti-class II antibodies, the expansion of the γδ T cells is affected indirectly. To address this possibility, IL-2 was added to cultures of PBMC, *P. falciparum* extract and anti-class II antibodies and the response of γδ T cells measured (Table 2). Addition of IL-2 to cultures in general increased the recovery of viable blasted cells regardless of the presence of parasite extract. In some cases (four of 10 donors) a significant fraction of the cells was Vγ9$^+$ T cells (e.g. C58 in Table 2). However, PBMC cultured in the presence of the parasite extract and IL-2 contained significantly more Vγ9$^+$ T cells, suggesting that the Vγ9$^+$ T-cell response was dependent upon the presence of antigen. When IL-2 was added to the cultures containing the anti-class II antibodies, the number of blasting Vγ9$^+$ T cells was largely restored to at least two-thirds of the level observed in the absence of antibodies (indicated in boldface in Table 2). The inhibition brought about by culture with anti-class II antibodies and the ability of IL-2 to circumvent this is summarized for all donors in Fig. 3a. In all cases anti-class II antibodies inhibited the response of Vγ9$^+$ T cells to *P. falciparum* extract by more than 70%. This inhibition was reversed by the addition of IL-2.

Inhibition of the Vγ9$^+$ γδ T-cell response by MHC class I antibodies cannot be reversed by IL-2

The ability of IL-2 to overcome the inhibition of the Vγ9$^+$ T-cell response to *P. falciparum* antigens indicated that Vγ9$^+$ T cells themselves do not recognize antigen and MHC class II molecules. Therefore we examined whether the Vγ9$^+$ T-cell response could be inhibited by antibodies to MHC class I. For these experiments three different antibodies specific for HLA-A, B and C molecules were used (Fig. 2b). As with the anti-class II antibodies, all three antibodies could inhibit the Vγ9$^+$ T-cell response to *P. falciparum* antigens (two representative examples are shown in Table 2).

With the highest concentration of antibody (12·5 μg/ml), flow cytometric analysis revealed that the percentage of blasting Vγ9$^+$ T cells was reduced from 57–74% to 10–22%.

Table 2. Inhibition of the $V\gamma9^+$ T-cell response to *P. falciparum* schizont antigen by anti-class I and class II antibodies

			Donor 58				Donor 62			
			Recovered viable cells ($\times10^3$)†		No. of cells within the blasting population ($\times10^3$)		Recovered viable cells ($\times10^3$)†		No. of cells within the the blasting population ($\times10^3$)	
Antigen	Antibodies added	IL-2	Total	Blasts‡	Vγ9	CD4	Total	Blasts‡	Vγ9	CD4
–		–	167	§	§	§	129	9	§	§
–		+	513	331	201	34	490	303	13	103
+	Control Ab	–	352	214	165	14	897	642	530	68
		+	1102	969	778	24	1530	1321	969	139
Anti-class II antibodies										
+	FMC1	–	172	19	**5**	8	114	17	**6**	4
		+	708	522	**438**	21	794	643	**567**	35
+	L243	–	122	27	**1**	13	512	82	**24**	12
		+	648	543	**411**	27	1658	1352	**1090**	99
+	HL-40	–	112	21	**4**	8	501	80	**18**	21
		+	737	642	**509**	17	2009	1664	**1454**	88
Anti-class I antibodies										
+	W6/32	–	107	49	4	21	154	10	53	27
		+	106	71	29	20	104	28	46	36
+	2A1	–	117	21	3	7	88	32	10	53
		+	164	102	60	8	150	104	28	46
+	B1-3D3	–	128	50	6	17	131	42	19	10
		+	125	66	35	13	246	66	15	23

* All antibodies were used at a final concentration of 12·5 μg/ml.
† Total number of viable cells recovered from triplicate microtitre wells.
‡ The proportion of blasting cells was defined by forward and large angle scatter on FACScan.
§ The number of cells within that category was too low to measure accurately.

The total number of blasting $V\gamma9^+$ T cells recovered from cultures of PBMC with anti-class I antibodies was reduced more than 50-fold for the donors shown in Table 2 (1·6–5·3 × 10^5 blasting $V\gamma9^+$ T cells in cultures reduced to 0·4–6·5 × 10^4 blasting cells recovered in the presence of anti-class I antibodies).

In contrast to the results obtained with the anti-class II antibodies, addition of exogenous IL-2 could not restore the $V\gamma9^+$ T-cell response after inhibition by the anti-class I antibodies. For the two donors in Table 2, both the total number of blasted cells and Vγ9-blasted cells remained unaltered by the addition of IL-2. This was true for all donors tested and is summarized in Fig. 3b. The three antibodies inhibited the T-cell response by 60–80% and this response was unaltered despite the addition of 10 U/ml of IL-2 to the cultures.

$CD4^+$ T cells can restore the $\gamma\delta$ T-cell response to *P. falciparum* antigens

In order to determine whether $\gamma\delta$ T cells required the presence of $CD4^+$ T cells for their response to *P. falciparum* antigens, graded numbers of highly purified $CD4^+$ T cells (over 99% purity) were cultured with enriched $\gamma\delta$ T cells in the presence of antigen and irradiated adherent cells. Under these conditions there was no $\gamma\delta$ T-cell response in the absence of $CD4^+$ T cells, and the proportion of blasting $V\gamma9^+$ T cells in the cultures increased with the addition of increasing numbers of $CD4^+$ T cells (Fig. 4).

DISCUSSION

These studies demonstrate that the *in vitro* response of $V\gamma9^+$ T cells from non-immune donors to *P. falciparum* schizont antigens is inhibited by both anti-class I and anti-class II antibodies. The ability of anti-MHC class II antibodies to inhibit the proliferative response of non-immune PBMC to *P. falciparum* antigen has been reported previously[13] and it was assumed at that time that the $\gamma\delta$ T cells used MHC class II molecules as their restriction elements. Later experiments on a limited number of donors,[25] however, indicated that a more complex series of reactions might be taking place within the culture. The experiments described here support the idea that $V\gamma9^+$ T cells themselves do not recognize MHC class II molecules but do require an MHC class II-restricted response taking place in the culture to provide the cytokines necessary for their activation. In support of this idea, a $CD4^+$ T-cell response of variable magnitude was observed in the cultures of PBMC from all donors, although these individuals had not been exposed to malaria. This response of non-exposed individuals has been observed previously[26] and is thought to be due to a response to determinants shared by *Plasmodium* and

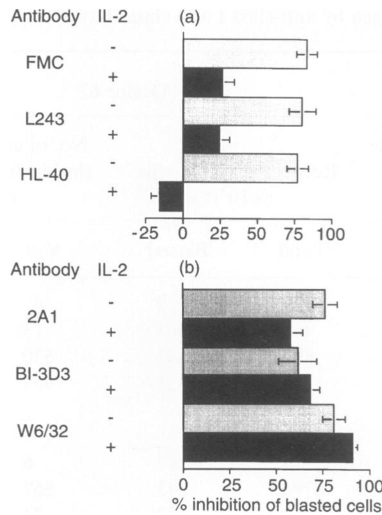

Figure 3. Inhibition of $\gamma\delta$ T-cell responses by anti-class II and anti-class I antibodies. The percentage specific inhibition of the blasting $V\gamma9^+$ response is shown. The error bars represent the SEM of the inhibition of the response of eight to 10 donors. (a) Reconstitution of the $V\gamma9^+$ cell response to *P. falciparum* antigens by IL-2 after inhibition by anti-class II antibodies. PBMC were cultured with *P. falciparum* culture supernatants in the presence of anti-class II antibodies, FMC 14, L243 and HL-40, at final concentrations of 12·5 μg/ml in the presence (▨) or absence (▢) of 10 U/ml recombinant IL-2. (b) Inability of IL-2 to restore the response of $V\gamma9^+$ T cells to *P. falciparum* antigens after inhibition by antibodies to MHC class I molecules. PBMC were cultured together with *P. falciparum* antigens and anti-class I antibodies, 2A1, BI-3D3 and W6/32, at final concentrations of 12·5 μg/ml in the presence (▨) or absence (▢) of 10 U/ml recombinant IL-2.

other pathogens or environmental antigens. The cytokines or costimulatory activity contributed by these cells may be responsible for facilitating growth and division of $V\gamma9^+$ T cells in culture. The ability of IL-2 to circumvent the inhibition of the $\gamma\delta$ T-cell response by anti-class II antibodies suggested this to be the case, and was further confirmed by the reconstitution of the response of purified $V\gamma9^+$ T cells to *P. falciparum* antigen with the addition of purified $CD4^+$ T cells to cultures. These data suggest two possibilities. First, the $V\gamma9^+$ T-cell response is not specific for malarial antigens and is simply

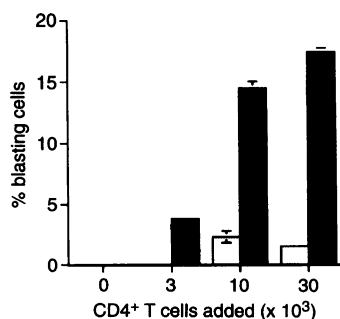

Figure 4. Blasting $\gamma\delta$ T-cell response to *P. falciparum* antigens in the presence of purified $CD4^+$ T cells. $\gamma\delta$ T cells (5×10^4 cells/ml) were cultured in with or without $CD4^+$ T cells (3×10^3–3×10^4 cells/well) in the presence (▨) or absence (▢) of 10% (v/v) *P. falciparum* culture supernatants. The SEM for duplicate cultures are shown.

due to the cytokines produced by the $CD4^+$ T-cell response. In line with this is the observation that human peripheral blood $V\gamma9^+$ T cells already express activation markers such as CD45RO[10] and higher levels of IL-2 receptor β chain[27] and thus may be readily activated *in vitro*.[27] A second possibility is that $V\gamma9^+$ T cells respond specifically to some parasite components, but require additionally an exogenous source of cytokines or costimulation for clonal expansion *in vitro*. Our data support the latter; the $V\gamma9^+$ T-cell response, although increased in the presence of IL-2 alone, was always higher in the presence of *P. falciparum* antigens. Preliminary experiments in which purified $\gamma\delta$ T cells respond to *P. falciparum* culture supernatants in the presence of IL-2 confirm the requirement for antigen in the cultures (S. Morris-Jones & J. Langhorne, unpublished observations).

The requirement for $CD4^+$ T cells or cytokines accords with other experimental systems. In mice, $CD4^+$ T cells are required for the $\gamma\delta$ T-cell expansion found with *P. chabaudi* infections.[28] Similarly, the human response to *Mycobacterium tuberculosis in vitro*, which also involves a prominent $V\gamma9^+$ cell proliferation, has also been shown to be dependent on $CD4^+$ T cells and IL-2.[29,30] Interestingly, these two studies found different mechanisms for the $CD4^+$ T-cell help. In one, soluble factors (primarily IL-2) mediated the $\gamma\delta$ T-cell response,[29] while in the other direct cell–cell contact with the $CD45RO^+$ $CD7^-$ subset of $CD4^+$ T cells was required to enable expansion.[30] Our experiments using recombinant IL-2 are in line with the former study. The reqirement of $\gamma\delta$ T cells for $CD4^+$ T cells or cytokines from these cells would appear to contradict the idea that $\gamma\delta$ T cells are part of the early 'non-adaptive' immune response. However, cytokines important for $\gamma\delta$ T-cell growth and differentiation are produced by non-lymphoid cells.[31] Furthermore, cytokines such as IL-2 may be present within the lymphoid organs as a result of other on-going immune responses. Thus activation of $\gamma\delta$ T cells could proceed without simultaneous activation of $CD4^+$ T cells.

The $V\gamma9^+$ T-cell response to *P. falciparum* was also inhibited by anti-MHC class I antibodies. In contrast to the experiments with anti-MHC class II antibodies, provision of exogenous IL-2 did not counteract the block even when added at high concentrations. This suggests that the antigen to which the $V\gamma9^+$ T cells are responding is associated with MHC class I molecules. The general nature of ligands for $\gamma\delta$ T cells is not certain, and although examples of MHC class I-restricted responses exist,[32,33] there are reports of unrestricted responses.[8,34] Attention has also focused on other potential presenting molecules, and there is evidence from mice[22,35,36] and humans[37,38] that these may be non-classical class I MHC molecules such as Qa-1 and CD1a, b, c, d. Although it has not been documented, it is possible that the anti-class I antibodies used in this study also recognize non-classical class I molecules. However, our preliminary studies with a limited number of donors with antibodies to CD1 molecules suggest that they are not the restriction elements for the $V\gamma9^+$ T-cell response to *P. falciparum* antigens (J. Langhorne, unpublished observations).

In addition to the lack of consensus about the MHC restriction of $\gamma\delta$ T cells, the nature of the determinant recognized is not clear. Some studies have demonstrated that $\gamma\delta$ T cells appear to be broadly cross-reactive.[37,39] Recognition of conserved molecules could account for this and there are reports of $\gamma\delta$ T-cell recognition of heat-shock proteins.[40–42]

More recently there have been descriptions of presentation of non-peptide lipid antigens by non-classical class I molecules.[22–24,43]

It is known that $V\gamma9^+$ T cells preferentially respond to *P. falciparum* antigens expressed on schizonts and released at schizont rupture.[17] It will be of importance to determine whether peptide or lipid-like molecules are the components responsible for the stimulation of γδ T cells in malaria and whether this is related to the malaria phospholipid toxins released at schizont rupture and responsible for macrophage activation and TNF release.[19]

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